Electronically Filed

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Brines *et al.*	Confirmation No.: 4194
Application No.: 10/185,841	Group Art Unit: 1647
Filed: June 26, 2002	Examiner: DeBerry, Regina M.
For: PROTECTION, RESTORATION AND ENHANCEMENT OF ERYTHROPOIETIN-RESPONSIVE CELLS, TISSUES AND ORGANS	Attorney Docket No.: 10165-015-999

SECOND DECLARATION OF MICHAEL L. BRINES, M.D., PH.D.

Sir:

I, MICHAEL L. BRINES, do hereby declare and state:

1. I am an inventor of the invention described and claimed in the above-identified patent application (hereinafter the "841 application"). I am presently Chief Scientific Officer at Warren Pharmaceuticals, Inc., licensee of the '841 application.

2. I have over thirty years of experience in biological research and clinical investigation. I am a certified member of the American Board of Internal Medicine. My academic and technical experience and honors, and a list of my publications, are set forth in my curriculum vitae, a copy of which is attached hereto as Appendix A.

3. I have read and am familiar with the '841 application, the pending claims and the outstanding Office Action. I understand that the technology of the '841 application relates to the use of erythropoietin ("EPO") and chemically modified forms of EPO for protecting, maintaining, enhancing or restoring the function or viability of cells, tissues and organs.[1] Such chemically modified forms of EPO can be EPO molecules that do

[1] For ease of reference, I will use the term "tissue-protection" instead of the phrase "protecting, maintaining, enhancing or restoring the function or viability of cells, tissues and organs." Likewise, the ability of a protein to protect, maintain, enhance or restore the function or viability of cells, tissues and organs will be referred to as its "tissue-protective" activity.

not increase hemoglobin concentration[2] but retain their tissue-protective activity. I have been informed and believe that the claims of the '841 application are subject to a rejection based on the contention that the '841 application does not provide sufficient evidence that chemically modified forms of EPO have tissue-protective effects in erythropoietin-responsive cells, tissues and organs as claimed.

4. I have been asked to provide evidence that chemically modified forms of EPO have tissue-protective effects in erythropoietin-responsive mammalian cells, tissues and organs.

5. In the following paragraphs I will present evidence that shows that chemically modified EPO molecules can provide tissue-protective activity in all tissues that coexpress the EPO receptor ("EpoR") and the common beta receptor ("cbR"). I will further present experimental data, citing a non-exhaustive list of examples from the literature, that chemically modified EPO has tissue protective effects in a wide range of different tissues.

I. THE TISSUE PROTECTIVE AND ERYTHROPOIETIC ACTIVITIES OF EPO ARE MEDIATED BY SEPARATE AND DISTINCT PATHWAYS

6. EPO provides tissue-protective activity via a separate pathway from the pathway it uses to exert is erythropoietic effects. (Brines *et al.*, 2004, PNAS 101: 14907-14912.) EPO exerts its erythropoietic effect via a classic EPO Receptor homodimer (the "classical EPO Receptor"), whereas the tissue-protective activity of EPO is due to the interaction of the molecule with a different receptor, known as the "Tissue-Protective Receptor Complex", a heteromer of the classical EPO Receptor and cbR, a signal-transducing subunit shared by the granulocyte-macrophage colony stimulating factor, and the IL-3 and IL-5 receptor. (Brines, 2004).

7. The chemically modified EPO molecules of the invention are non-erythropoietic yet retain tissue protective activity. These chemically modified EPO molecules are tissue protective because they retain their ability to interact with the Tissue-Protective Receptor Complex, but have reduced erythropoietic activity compared with native EPO, e.g., by losing its ability to interact with the classical EPO Receptor. Based on these

[2] Molecules that do not increase hemoglobin concentration in a mammal will be referred to as non-erythropoietic.

properties, the chemically modified, non-erythropoietic forms of EPO can be expected to provide tissue-protection in any tissue which has a Tissue-Protective Receptor Complex, *i.e.* any erythropoietin-receptive tissue.

8. Therefore, chemically modified forms of EPO can be expected to exert tissue-protective effects in all tissues that coexpress the EpoR and the cbR. A detailed list of tissues expressing Common Beta Receptor is attached as Appendix B. Appendix B also contains a non-exhaustive list of references that describe EpoR-expressing cells, tissues, or organs. For example, representative cell types demonstrated to express the EpoR include, but are not limited to, endothelial cells, myocardiocytes, macrophages, retinal cells, cells of the adrenal cortex and medulla, small bowel, spleen, liver, kidney and lung, as well as cells of the central nervous system, such as neurons and glial cells, and astrocytes. Thus, I believe that the tissue protective activity of EPO, which is mediated through the Tissue-Protective Receptor Complex, may be found in all tissues.

9. In the paragraphs 10 to 23, below, I describe experiments from the literature that demonstrate that EPO and chemically modified forms of EPO, *e.g.*, carbamylated EPO ("CEPO"), showed both tissue-protective activity in a variety of tissue types.

II. CENTRAL NERVOUS SYSTEM

10. Numerous studies have corroborated and extended the applicant's discovery that carbamylated recombinant human erythropoietin ("rhCEPO") shows tissue-protective activity in the brain. For example, Wang *et al.* (Brit. J. Pharmacol. 2007, 151: 1337-1384) have shown, using a rat model of focal cerebral ischemia, that carbamylated recombinant human EPO significantly reduced the cortical infarct volume and reduced neurological impairment. Wang *et al.* also showed in analogous experiments that also recombinant human EPO ("rhEPO") significantly reduced the cortical infarct volume and reduced neurological impairment. These data indicate that rhCEPO, as well as rhEPO, show anti-inflammatory and anti-apoptotic effects. Based on these studies, I conclude that CEPO shows tissue-protective activity in brain tissue.

11. Chemically modified forms of EPO have also been shown to provide tissue-protective activity in the spinal cord. Data presented in Savino *et al.* (J.

Neuroimmunol. 2006, 172: 27-37) showed that the EPO derivatives CEPO and asialo EPO, as well as rhEPO, are active in a chronic model of experimental autoimmune encephalomyelitis (EAE). The action of CEPO was associated with a decrease in the production of inflammatory cytokines in the spinal cord and peripheral lymphocytes. Savino *et al.* shows an anti-inflammatory effect of CEPO in spinal cord tissue. These studies clearly demonstrate that chemically modified forms of EPO provide tissue-protective activity in spinal cord tissue.

III. PERIPHERAL NERVOUS SYSTEM

12. Chemically modified EPO has also been shown to provide tissue-protective activity in peripheral nerve tissue. Bianchi *et al.* (Clin. Canc. Res. 2006, 12: 2607-2612) evaluated EPO and CEPO in an experimental model of peripheral neurotoxicity induced by cisplatin that closely resembles cisplatin neurotoxicity in humans. Cisplatin given to Wistar rats significantly lowered their growth rate, with slower sensory nerve conduction velocity and reduced intraepidermal nerve fiber density. Coadministration of cisplatin and EPO or cisplatin and CEPO partially, but significantly, prevented the sensory nerve conduction velocity reduction. Both molecules preserved intraepidermal nerve fiber density. Based on these studies, it is clear that CEPO shows tissue-protective activity in peripheral nerve tissue.

IV. OTHER TISSUES

13. Chemically modified EPO has also been shown to provide tissue-protective activity in heart tissue. Moon *et al.* (J. Pharmacol. Exp. Ther. 2006, 316: 999-1005) showed in an experimental model of myocardial infarction induced by permanent ligation of a coronary artery in rats that a single bolus injection of 30 ug/kg b.wt. of CEPO, similarly to EPO, immediately after coronary ligation reduced apoptosis in the myocardial area at risk, examined 24h later, by 50%. These data indicate that CEPO, as well as EPO, shows anti-apoptotic effects in heart tissue. Based on this study, both CEPO and EPO show tissue-protective activity in heart tissue.

14. Chemically modified EPO has further been shown to provide tissue-protective activity in the kidney. Kitamura *et al.* (Nephrol. Dial. Transplant. 2008, 0: 1-8) evaluated the therapeutic effects of CEPO using a rat unilateral ureteral obstruction model.

In this model, CEPO decreased tubular apoptosis and alpha-smooth muscle actin expression in the absence of polycythaemia, while the untreated obstructed kidneys exhibited increased tubular apoptosis with expanded alpha-smooth muscle actin expression. While EPO treatment similarly inhibited tubular apoptosis and alpha-smooth muscle actin expression, EPO treatment increased hemoglobin concentrations and induced a wedge-shaped infarction. These data indicate that CEPO, as well as EPO, shows anti-apoptotic effects in kidney tissue. Based on these studies, EPO and CEPO shows tissue-protective activity in kidney tissue.

15. Chemically modified EPO has also been shown to provide tissue-protective activity in skin tissue. Brines *et al.*, (WO2005/032467, at Example 5, p. 37) demonstrated the use of an ischemic wound flap model to determine the effect of CEPO on ischemic skin flap wound recovery. The rats that received CEPO had a greater percentage of the wound healed than those treated with saline for the same period. This data demonstrates that CEPO decreases wound size and accelerates healing. Thus, CEPO shows tissue-protective activity in skin tissue.

16. EPO can also provide tissue protection of the cochlea. Mammalian auditory hair cells are unable to regenerate and can be irreversibly damaged by various agents, including gentamicin. Monge *et al.* (Laryngoscope 2006, 116: 312-316) presented data that showed a dose-dependent protective effect of EPO on gentamicin-damaged hair cells *in vitro*. The authors concluded that decreased hair cell loss in EPO-treated organs of Corti that had been exposed to gentamicin provides evidence for a protective effect of EPO in aminoglycoside-induced hair cell death. As described above, since the tissue-protective effect is attributed to the interaction of EPO with the Tissue-Protective Receptor Complex, this experiment indicates the presence of functional Tissue-Protective Receptor Complex in the cochlea. Since EPO shows a tissue protective effect in this experiment, it is expected that a chemically modified, non-erythropoietic form of EPO having tissue protective activity would show the same tissue protective properties in the cochlea. Based on these studies, I can conclude that chemically modified forms of EPO can be expected to show tissue-protective activity in the cochlea.

17. EPO can also show tissue-protective activity in striated muscle tissue. Contaldo *et al.* (Am. J. Physiol. Heart Circ. Physiol. 2007, 293: H274-H283) investigated the effect of EPO in ischemia-reperfusion (I/R)-induced microcirculatory dysfunctions. The

study demonstrated that EPO effectively attenuates I/R injury by preserving nutritive perfusion, reducing leukocytic inflammation and inducing new vessel formation. The authors concluded that EPO protects the striated muscle microcirculation of the dorsal skinfold from postischemic injury in mice. Because the tissue-protective effect is attributed to the interaction of EPO with the Tissue-Protective Receptor Complex, this experiment demonstrates the presence of functional Tissue-Protective Receptor Complex in striated muscle tissue. Since EPO shows a tissue protective effect in this experiment, it is expected that a chemically modified, non-erythropoietic form of EPO having tissue protective activity would show the same tissue protective properties in striated muscle tissue. Based on these studies, I conclude that chemically modified forms of EPO can show tissue-protective activity in striated muscle tissue.

18. Chemically modified forms of EPO can show tissue-protective activity in endothelial tissue. Employing an elevated glucose model for endothelial cells, Chong *et al.* (Curr. Neurovasc. Res. 2007, 4: 194-204) illustrated that a final glucose concentration of 25nM over a 48h course leads to a significant loss in cell survival and correspondingly a significant increase in genomic DNA degradation when compared to control endothelial cells. Administration of EPO significantly enhanced endothelial cell survival during elevated glucose. EPO also blocked apoptotic DNA degradation in endothelial cells during elevated glucose similar to alternate models of oxidative stress in cardiac and vascular cell models. The authors showed that EPO protects endothelial cells from apoptosis. Since tissue-protective effect of EPO results from the interaction of EPO with the Tissue-Protective Receptor Complex. This experiment demonstrates the presence of the Tissue-Protective Receptor Complex in endothelial tissue, and thus, chemically modified, non-erythropoietic forms of EPO having tissue protective activity would be expected to show the same tissue protective properties in endothelial tissues. Based on these studies, I conclude that chemically modified forms of EPO can show tissue-protective activity in endothelial tissue.

19. EPO has also been shown to provide tissue-protective activity in hair follicles. Bodo *et al.* (FASEB J. 2007, 21: 3346-3354) used organ-cultured hair follicles to assess the effects of EPO in the presence/absence of classical apoptosis-inducing chemotherapeutic agents. Bodo *et al.* demonstrated that EPO significantly down-regulates chemotherapy-induced intrafollicular apoptosis. Based on EPO's productive interaction with the Tissue-Protective Receptor Complex in this experiment, a chemically modified, non-

erythropoietic, tissue protective form of EPO would be expected to have the same tissue-protective properties in hair follicles. Based on this study, I conclude that chemically modified forms of EPO would have tissue-protective activity in hair follicles.

20. EPO can also provide tissue-protective activity in bone tissue. Holstein *et al.* (Life Sci. 2007: 893-900) investigated the effect of EPO treatment on bone healing in a murine closed femur fracture model. EPO-treated animals showed a higher torsional stiffness and an increased callus density when compared to vehicle-treated controls. Accordingly, the histomorphometric examination revealed an increased fraction of mineralized bone and osteoid. The authors concluded that EPO is involved in the process of early endochondral ossification, enhancing the transition of soft callus to hard callus. Since EPO shows a tissue protective effect in this experiment, it can be concluded that chemically modified, non-erythropoietic, tissue-protective forms of EPO would show the same tissue protective properties in bone tissue. Based on this reasoning, I conclude that chemically modified forms of EPO can provide tissue-protective activity in bone tissue.

21. EPO can also provide tissue-protective activity in intestinal tissue. Guneli *et al.* (Mol. Med. 2007, 13, 509-517) investigated whether EPO could prevent intestinal tissue injury in Wistar rats induced by ischemia-reperfusion (I/R). Following histological assessment using a microscopic scoring system to evaluate the I/R injury in the intestinal tissue the authors concluded that their study established that a high single dose of rhEPO administered both before ischemia and at the onset of reperfusion protected the intestinal tissue against I/R injury. They further state that data from their study demonstrate that antiapoptotic, antioxidative and anti-inflammatory properties seem to be related to the EPO-mediated protective effect against I/R injury. Because the tissue-protective effect in this example is attributed to the interaction of EPO with the Tissue-Protective Receptor Complex and because EPO shows a tissue protective effect in this experiment, a chemically modified, non-erythropoietic, tissue protective form of EPO would also be expected to provide tissue protection in the intestine. Based on these studies, I conclude that chemically modified forms of EPO can be used to provide tissue-protective activity in intestinal tissue.

22. EPO has also been shown to provide tissue-protective activity in lung tissue. Tascilar *et al.* (World J. Gastroenterol. 2007, 13: 6172-6182) investigated the effect of exogenous EPO administration on acute lung injury in an experimental model of sodium

taurodeoxycholate-induced acute necrotizing pancreatitis. The study shows that the mean pleural effusion volume, calculated lung/body weight ratio, serum IL-6 and lung tissue malondialdehyde levels were significantly lower in EPO groups than in ANP groups. Tascilar *et al.* concluded that histopathological evaluation confirmed the improvement in lung injury parameters after exogenous administration of EPO. Since the tissue-protective effect in this example is attributed to the interaction of EPO with the Tissue-Protective Receptor Complex, I conclude that a chemically modified, tissue-protective, non-erythropoietic form of EPO would also provide tissue protection in the lung. Based on this reasoning, I conclude that chemically modified forms of EPO can be used to provide provide tissue protection in lung tissue.

23. EPO can also provide tissue-protective activity in liver tissue. Yazihan *et al.* (Turk. J. Gastroenterol. 2007, 18: 239-244) used a human hepatocyte cell line for assays to determine whether EPO treatment decreases H_2O_2-induced toxicity. Yazihan *et al.* reported that EPO treatment significantly increased cell number at the 24^{th} and 48^{th} hour compared to the control group. H_2O_2 application induced apoptosis and lactate dehydrogenase release from Hep3B cells and decreased cell number. EPO prevented H_2O_2 toxicity in hepatocytes. Since tissue protection is attributed to the interaction of EPO with the Tissue-Protective Receptor Complex, a chemically modified non-erythropoietic, tissue protective form of EPO would also provide tissue protection in the liver. Based on these studies, I conclude that chemically modified forms of EPO can be used to provide tissue-protective activity in liver tissue.

V. CONCLUSION

24. In summary, I have presented experimental data, citing a non-exhaustive list of examples from the literature, that chemically modified EPO has tissue protective effects in a wide range of mammalian tissues. Thus, there is ample evidence from the data presented in the literature that chemically modified, non-erythropoietic, tissue protective forms of EPO can exert tissue-protective activity in any erythropoietin-responsive tissue, *i.e.*, any tissues that expresses an Tissue-Protective Receptor Complex.

25. I declare further that all statements made in this Declaration of my own knowledge are true, that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

February 20, 2008
Date

Michael L. Brines
Michael L. Brines

Attachments:

Appendix A: Curriculum Vitae of Dr. Michael L. Brines, M.D., Ph.D.
Appendix B: References showing tissues that express the EpoR and/or cbR
Appendix C: Cited References